DOPA-Negative Melanocytes in the Outer Root Sheath of Human Hair Follicles Express Premelanosomal Antigens But Not a Melanosomal Antigen or the Melanosome-Associated Glycoproteins Tyrosinase, TRP-1, and TRP-2

Tatsuya Horikawa,* David A. Norris,*‡ Thomas W. Johnson,§ Tamara Zekman,* Nancy Dunscomb,† Scott D. Bennion,§ Ronald L. Jackson,§ and Joseph G. Morelli*

*Department of Dermatology and †Department of Pathology Cancer Core, University of Colorado School of Medicine and ‡Department of Veterans Affairs Hospital; and §Department of Dermatology, Fitzsimons Army Medical Center, Denver, Colorado, U.S.A.

It is believed that DOPA-negative melanocytes in the outer root sheath of the human hair follicle are activated, become identifiable by DOPA staining, and migrate into the epidermis during the repigmenting phase of vitiligo. These cells are difficult to identify, however, and otherwise have not been characterized. These cells are readily identified by immunofluorescence, immunohistochemistry, and immunoelectronmicroscopy using the antibodies NKI/beteb and A4F11, which recognize premelanosome-related antigens. The majority of the outer root sheath melanocytes were found in the mid to the upper portion of the hair follicle. Double staining revealed that these cells were distinct from HLA-DR–bearing dendritic cells. Further immunohistochemical investigation using α-PEP-7, α-PEP-1, or TMH-1 and α-PEP-8 antibodies revealed that outer root sheath melanocytes cannot be identified by antibodies to tyrosinase, TRP-1, or TRP-2, respectively. These cells also did not react with HMB45 antibody, which recognizes a melanosome-associated cytoplasmic antigen. We believe that the inactive outer root sheath melanocytes contain some of the early structural proteins but not any of the enzymatic proteins necessary for melanogenesis. Therefore, activation is the process whereby outer root sheath melanocytes acquire all of the structural and enzymatic proteins necessary for melanogenesis. ***Key words: vitiligo/repigmentation/outer root sheath/premelanosome. J Invest Dermatol 106:28–35, 1996***

It has been demonstrated that human hair follicle outer root sheath (ORS) melanocytes are identifiable by toluidine blue staining but not by DOPA staining [1,2], and become identifiable by DOPA staining following dermabrasion or ultraviolet irradiation [3,4]. It is believed that ORS melanocytes are the reservoir for repopulation of epidermal melanocytes during re-epithelization following loss of epidermis and for repigmentation in vitiligo.

Vitiligo is characterized by the destruction of epidermal melanocytes leading to areas of depigmentation [5–7]. Repigmentation of vitiligo is usually seen in a perifollicular manner [8]. During this process it is thought that inactive ORS-melanocytes become activated, proliferate, and migrate into depigmented epidermis [9]. Thus, ORS melanocytes play an important role in repigmentation of vitiligo. However, these cells are difficult to identify and have been poorly characterized [9–11].

Recently it has been shown that not only tyrosinase but also tyrosinase-related protein-1 (TRP-1), tyrosinase-related protein-2 (TRP-2), and the silver locus/Pmel-17 protein play important roles in melanin synthesis by pigment cells [12–14]. We have refined the identification of ORS melanocytes by using antibodies to premelanosome-associated antigens and further characterized these cells.

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Reprint requests to: Dr. Joseph G. Morelli, Department of Dermatology, University of Colorado School of Medicine, 4200 East Ninth Avenue B-153, Denver, Colorado 8026.

Abbreviation: TRP-1, tyrosinase related protein-1.

MATERIALS AND METHODS

Hair Samples Human hairs were obtained by plucking from three Asian adult healthy volunteers (two men, 37 years old and 40 years old, and a 35-year-old woman). Scalp skins were obtained from plastic surgery (brown hair) and autopsy (black hair) samples with approval of the Institutional Review Board for Use of Human Subjects in Research. Scalp skins were incubated with 2 M NaBr for 1 to 2 h at 37°C, then the epidermis and the hair follicles were separated from the dermis. Using this separation technique, hair follicles were completely preserved from the bulbar to the infundibular portion.

Antibodies For the immunohistochemical and immunofluorescent studies, the following antibodies were used. 1) NKI/beteb (Accurate Chemical and Scientific Corporation, Westbury, NY), which recognizes 100- and 7-kD molecular weight antigen(s) in melanosomes, premelanosomes, or premelanosome-like structures [15]. 2) A4F11 recognizes 17-, 18-, and 50-kD antigen(s) in association with premelanosomes and melanosomes [16] and was a kind gift from Dr. K. Hayashibe (Department of Dermatology, Kobe University School of Medicine, Japan). 3) HMB45 (Enzo Diagnostic, Syosset, NY), which recognizes a 10-kD melanosome-associ-

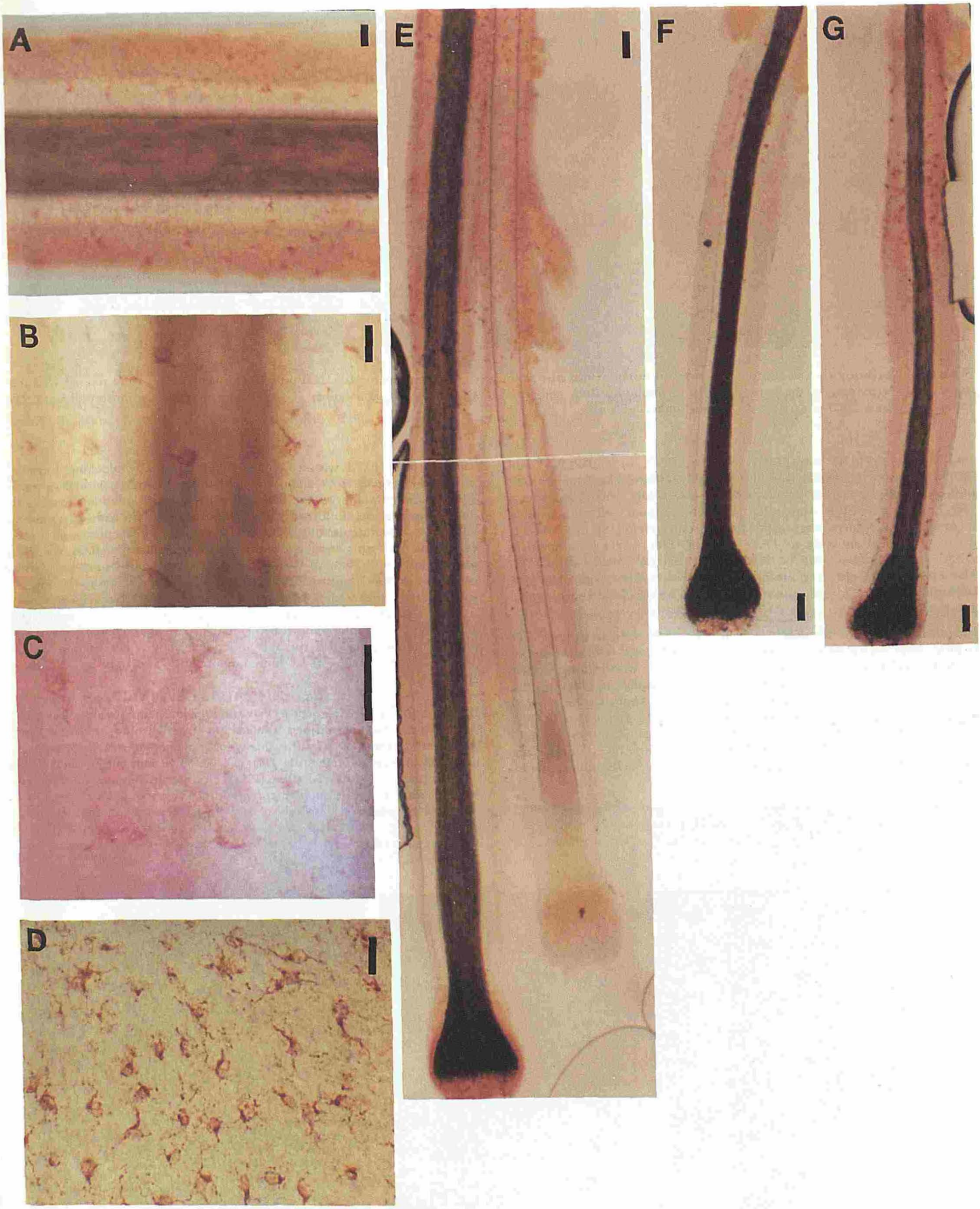

Figure 1. ORS melanocytes are identified by antibodies to premelanosomal antigens. Hair follicles were stained with streptavidin-peroxidase using monoclonal antibodies NKI/beteb or A4F11. Plucked hairs show NKI/beteb- (*A, B*) and A4F11- (*C*) positive dendritic cells. Epidermal melanocytes are shown as positive control (*D*) after incubation with NKI/beteb. NaBr-separated hair follicles show NKI/beteb-positive cells in the ORS, hair bulbs, and sebaceous glands (*E, F, G*). Most of the hair follicles contained NKI/positive cells predominantly in the middle to upper portions (*E*). In some of the hair follicles only a few positive cells could be identified (*F*). In a few hairs melanocytes were also found in the lowest portion (*G*) of the follicle. NKI/beteb-positive cells were also observed in the ORS of a senile white hair (*E*). *Bars*, 25 μm for *A, B, C, and D*, or 100 μm for *E, F, and G*.

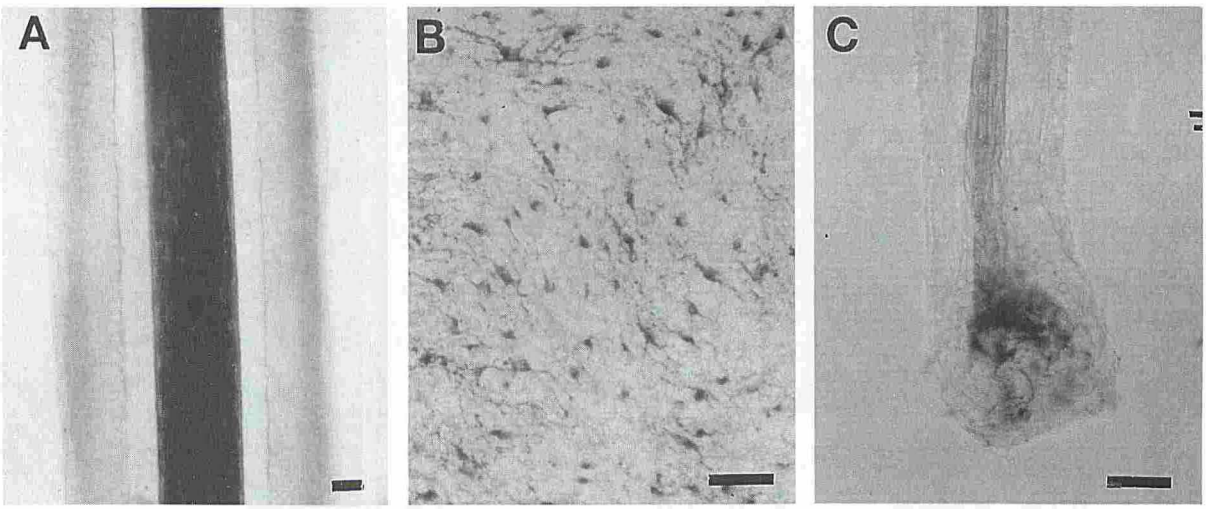

Figure 2. Antibody to a melanosome-related antigen did not react with ORS melanocytes. NaBr-separated hair follicles were stained with the streptavidin-peroxidase method using HMB-45 antibody. Both epidermal interfollicular (*B*) and bulbar (*C*) melanocytes were demonstrated with this antibody, but not ORS melanocytes (*A*). *Bars* indicate 50 μm.

ated antigen [17]. 4) Anti-PEP-1, 5) anti-PEP-7, 6) anti-PEP-8, and 7) TMH-1 were kind gifts from Dr. V. J. Hearing (Laboratory of Cell Biology, National Cancer Institute, Bethesda, MD). Anti–PEP-1, anti–PEP-2, and anti–PEP-8 were raised in rabbits immunized with peptide fragment of TRP-1, tyrosinase, and TRP-2, respectively [18]. 8) TMH-1 is a rat monoclonal antibody to TRP-1 [19]. 9) Flourescein isothiocyanate (FITC)–conjugated anti–HLA-DR antibodies (Becton Dickinson, Bedford, MA), 10) FITC-conjugated anti-mouse IgG (Fisher, Pittsburgh, PA), [11] rhodamine-conjugated anti-mouse IgG (Fisher), and [12] FITC-conjugated anti-rat IgG (Sigma, St. Louis, MO) were purchased.

Immunofluorescent Studies Hair samples were stained without sectioning. After cold acetone fixation, hairs were incubated with the primary antibody for 1 h at room temperature. Samples were washed, then incubated with FITC-conjugated secondary antibodies specific to the primary antibody. For double-staining studies, plucked hairs were incubated with mouse monoclonal antibodies, NKI/beteb (1:40 dilution) or A4F11 (1:1000 dilution), and then with rhodamine-conjugated anti-mouse IgG (goat, 1:100 dilution, Fisher). After blocking with mouse IgG (Fisher), hairs were then incubated with FITC-conjugated anti–HLA-DR mouse monoclonal antibodies (1:50 dilution).

Immunohistochemical Studies The streptavidin-peroxidase method was performed for immunohistochemical studies. For staining using NKI/beteb, A4F11, or HMB45, hair samples without sectioning or cryostat sections were fixed with cold acetone, followed by blocking intrinsic peroxidase with 3% H_2O_2 and nonspecific antibody binding with 8% normal swine serum. Samples were incubated with primary antibodies for 1 h at room temperature, followed by a 30-min incubation with biotinylated anti-mouse immunoglobulins (DAKO Corporation, Carpenteria, CA). After incubation with streptavidin-peroxidase complex (DAKO), samples were treated with 3-amino-9-ethylcarbazole chromogen. For staining with α-PEP-1, α-PEP-7, and α-PEP-8, 10% formaldehyde fixation (10 min at room temperature) with 0.02% saponin treatment or 2% paraformaldehyde fixation followed by 0.1% Triton-X treatment were done. Biotinylated anti-rabbit immunoglobulins (DAKO) replaced the biotinylated anti-mouse immunoglobulins in the method described above. For staining with TMH-1, biotinylated anti-rat immunoglobulins were used as secondary antibodies.

Immunoelectron Microscopy Immunoelectron microscopy was performed using the saponin-permeabilization technique [16]. Plucked hairs were pre-fixed with 4% paraformaldehyde supplemented with 0.1% saponin in 0.1 M phosphate buffer (pH 7.4) at 4°C for 30 min and washed three times in phosphate-buffered saline (PBS). Nonspecific binding was blocked by incubating with PBS supplemented with 0.1% saponin, 1% normal goat serum, 0.1% gelatin, 0.05% NaN_3, and 1% bovine serum albumin (washing buffer) for 3 h. The hairs were then incubated with NKI/beteb (mouse monoclonal antibody) or with purified mouse IgG (purified mouse IgG,

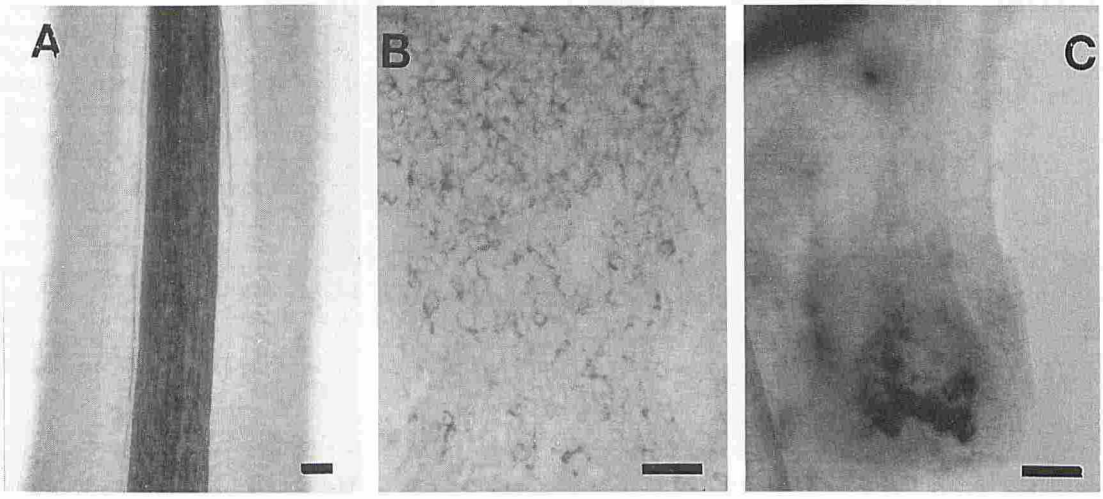

Figure 3. Antibodies to TRP-2 did not react with ORS melanocytes. NaBr-separated hair follicles were stained with the streptavidin-peroxidase method using anti PEP-8. Both epidermal (*B*) and bulbar (*C*) melanocytes showed positive but not ORS melanocytes (*A*). *Bars* indicate 50 μm.

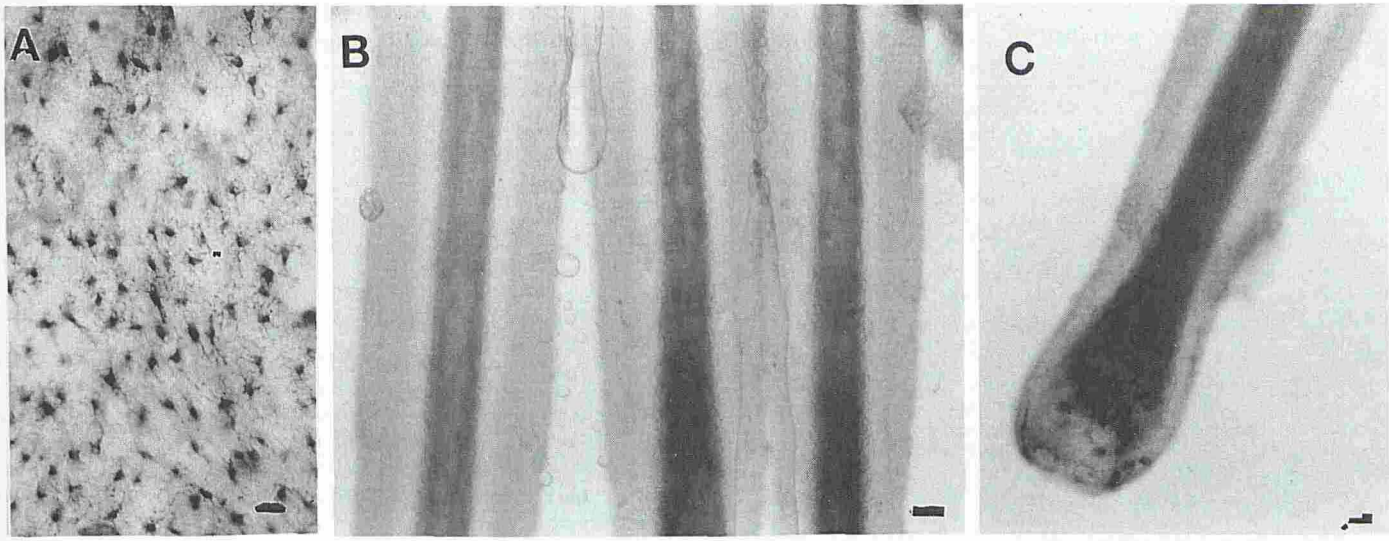

Figure 4. ORS melanocytes cannot be identified by DOPA staining. Dopa-positive melanocytes are seen in interfollicular epidermis (*A*) and in the hair bulb (*B*) of plucked hair, but not in the ORS of plucked hairs (*C*). *Bars* indicate 50 μm.

Sigma) in washing buffer without saponin at 4°C overnight. After three washes, the hairs were incubated with Fab′ fragment of goat anti-mouse IgG conjugated with colloidal gold particles (1.4-nm gold particles, Nanoprobe, Inc., Stony Brook, NY) at 4°C overnight. The hairs were then washed in PBS and post-fixed in half-strength Karnovsky fixative for an hour at room temperature. Silver enhancement was done according to the method described in the HQ Silver Enhancement Kit (Nanoprobe). The hairs were then processed by standard embedding procedures.

Other Stains For DOPA staining, unsectioned hairs were incubated with 0.1% L-DOPA in PBS (pH 7.4) overnight at 37°C. Toluidine blue and DOPA-premelanin staining were performed as described by Kiernan [19] and Mishima [20].

Number and Distribution of ORS Melanocytes Thirty-two plucked hairs were stained immunohistochemically with A4F11. The length of ORS was measured under microscopy and each ORS was divided into three sections from the top to the bottom of the ORS. Cells staining with A4F11 were counted on one side of the hair follicle and this number was multiplied by two to give an estimate of the total number for each hair.

RESULTS

ORS Melanocytes Were Identified by Antibodies to Pre-melanosome-Related Antigens But Not by Antibodies to a Melanosome-Related Antigen, Tyrosinase, TRP-1, or TRP-2 ORS melanocytes were demonstrated immunohistochemically by antibodies, NKI/beteb **(Fig 1*A*,*B*,*E*–*G*)** and A4F11 **(Fig 1*C*)**. Both round and dendritic shaped cells were seen. Epidermal melanocytes were used as the positive control **(Fig 1*D*)**. The staining pattern observed using these antibodies was granular and cytoplasmic **(Fig 1*B*,*C*)**. The negative control was an isotype-matched antibody (anti-human IgA mouse monoclonal antibody) (not shown). Hair bulb melanocytes also stained using NKI/beteb and A4F11 (1E-G).

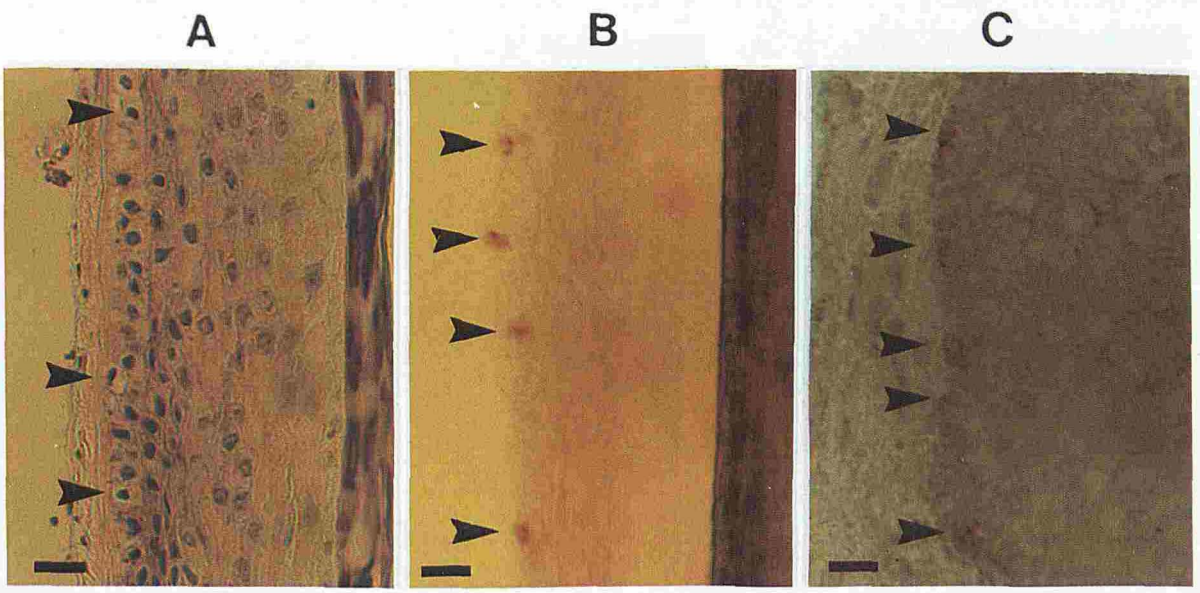

Figure 5. ORS melanocytes are easier to identify using NKI/beteb antibody rather than toluidine blue staining. *A*) Paraffin-embedded sections were stained with toluidine blue. *Arrowheads* show cells with clear cytoplasm and dark stained nuclei, indicating ORS melanocytes, although questionable cells with clear cytoplasms are seen between the arrows. *B*) Plucked hairs were stained with the streptoavidin-peroxidase method using NKI/beteb. The photograph was taken focusing on the edge of the hair follicle ORS, showing the ORS melanocytes lining the edge of the root sheath. *Arrowheads* show NKI/beteb-positive cells in the ORS. *C*) Cryostat sections of scalp hair follicles were stained with the streptoavidin-peroxidase method using NKI/beteb. *Arrowheads* show NKI/beteb-positive cells in the ORS. *Bars*, 25 μm.

Table I. NKI/beteb and A4F11 But Not HMB45, Anti-PEP-1, Anti-PEP-7, Anti-PEP-8, or TMH-1 Antibodies Identified ORS Melanocytes[a]

	Plucked hair	NaBr-Separated Hair		
Antibodies	ORS	Bulb	ORS	Epidermis
NKI/beteb	++	+	++	++
A4F11	++	+	++	++
HMB45	−	+	−	++
Anti-PEP-1	−	+	−	+
Anti-PEP-7	−	+	−	+
Anti-PEP-8	−	+	−	+
TMH-1	−	ND[b]	ND	+
DOPA	−	+	−	++

[a] Plucked hairs of NaBr-separated hair follicles were stained immunohistochemically using various kinds of antibodies. Staining results were represented as −, negative; +, positive; and ++, strongly positive.
[b] ND, not done.

Table II. A4F11-Positive ORS Melanocytes Are Located Predominantly in the Middle and Upper Portions of the ORS

ORS Portions	Number of Cells[a]
Lower portion	1.4 ± 0.5
Middle portion	43.9 ± 4.6
Upper portion	26.8 ± 3.0
Total	72.3 ± 6.4

[a] Data are represented as the mean ± SEM for 32 individual plucked hairs.

ORS melanocytes did not stain using HMB45 antibody, which binds to a melanosome-associated cytoplasmic antigen **(Fig 2*A*)**. Epidermal **(Fig 2*B*)** and hair bulb melanocytes **(Fig 2*C*)** did stain using this antibody. Interestingly, the hair bulb of senile white hairs did not stain with either NKI/beteb **(Fig 1*E*)** or HMB45 (not shown). However, NKI/beteb-positive cells were demonstrated in the ORS of white hairs **(Fig 1*E*)**.

Hair follicle ORS melanocytes were not identified using antibodies against the melanocyte enzymatic glycoproteins, tyrosinase, TRP-1 and TRP-2, although epidermal and hair bulb melanocytes were stained using these antibodies **(Fig 3)**. Neither DOPA **(Fig 4*C*)** nor DOPA-premelanin identified ORS melanocytes (data not shown), although both hair bulb and epidermal melanocytes were positively stained **(Fig 4*A*,*B*)**. Toluidine blue staining identified melanocytes along the basal layer of ORS, but these cells are difficult to recognize using this method **(Fig 5*A*)** compared to NKI/beteb staining **(Fig 5*B*,*C*)**. The results of the immunofluorescence and immunohistochemistry are summarized in **Table I**.

ORS Melanocytes Identified by NKI/beteb and A4F11 Are Distinct from HLA-DR Bearing Dendritic Cells Because HLA-DR bearing Langerhans cells are also dendritic and reside in the ORS, we performed double staining to differentiate the Langerhans cells from ORS melanocytes. Double staining revealed that ORS cells identified by NKI/beteb **(Fig 6*B*)** were distinct from HLA-DR bearing Langerhans cells **(Fig 6*C*)**. ORS cells detected by A4F11 were identical to those detected by NKI/beteb and were also distinct from HLA-DR bearing cells (not shown).

Distribution of ORS Melanocytes The distribution patterns of ORS melanocytes were variable. Most hair follicles had ORS melanocytes predominantly in the middle to upper portions **(Fig 1*E*)**. The number of ORS melanocytes per hair follicle was also quite variable **(Fig 1*E–G*)**. A few hair follicles showed ORS melanocytes both in the middle to upper portions and also in the bulbar to the lowest portions of ORS **(Fig 1*G*)**. Positive cells were seen in the sebaceous glands and their ducts **(Fig 1*E*)**. To quantify the distribution of ORS melanocytes, 32 plucked hairs were stained with A4F11 and the positive cells in the ORS were counted under microscopy. Each ORS was divided into upper, middle, and lower portions according to their length for the assessment of the distribution of ORS melanocytes. The majority of ORS melanocytes were located in the middle to upper portion of the ORS of hair follicles **(Table II)**. Cryostat sections also showed the same distribution of NKI/beteb-positive ORS melanocytes (not shown).

Immunoelectron Microscopy Using immunoelectron microscopy, we were able to verify that NKI/beteb localized to small round organelles in the cytoplasm or ORS melanocytes, but not the adjacent keratinocytes **(Fig 7)**. In **Fig 7*A***, an ORS melanocyte (nucleus marker N) is seen adjacent to an ORS keratinocyte

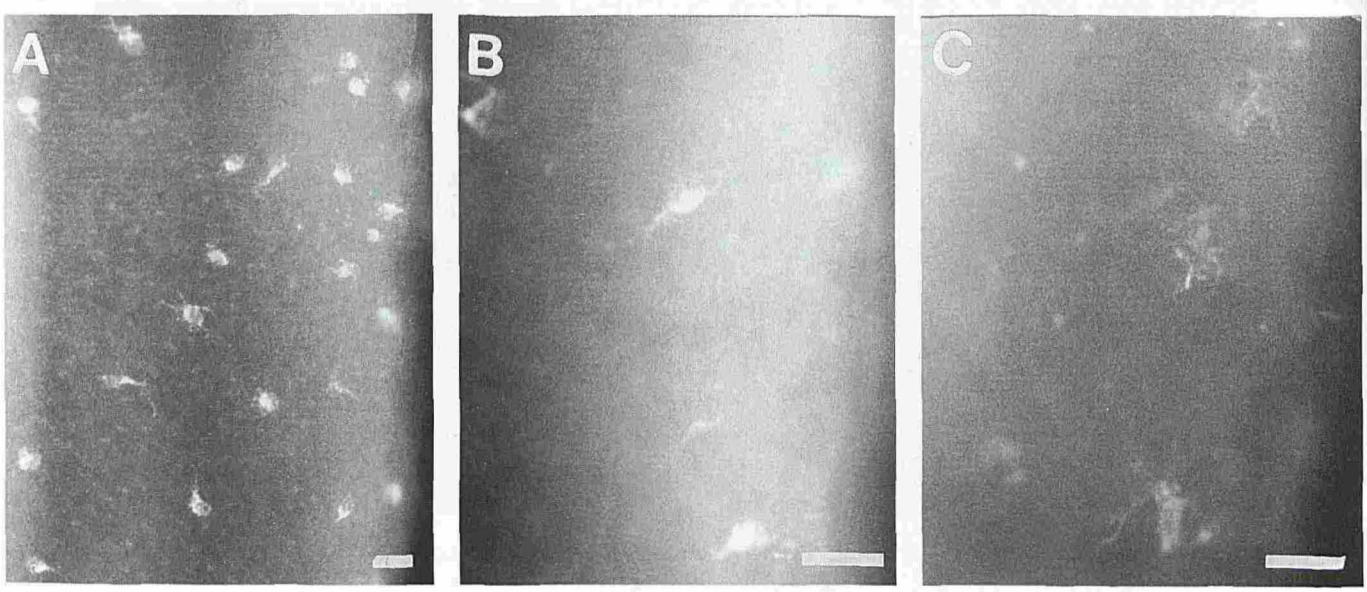

Figure 6. NKI/beteb-positive dendritic cells are distinct from HLA-DR bearing Langerhans cells. Plucked hairs were double-stained with NKI/beteb and FITC-conjugated anti-HLA-DR antibody. Rhodamine-conjugated secondary antibodies were used to visualize NKI/beteb-positive cells (*A*,*B*). NKI/beteb-staining cells are distinct from HLA-DR–positive cells (*C*). *B* and *C* are the same field with different filters. *Bars*, 25 μm.

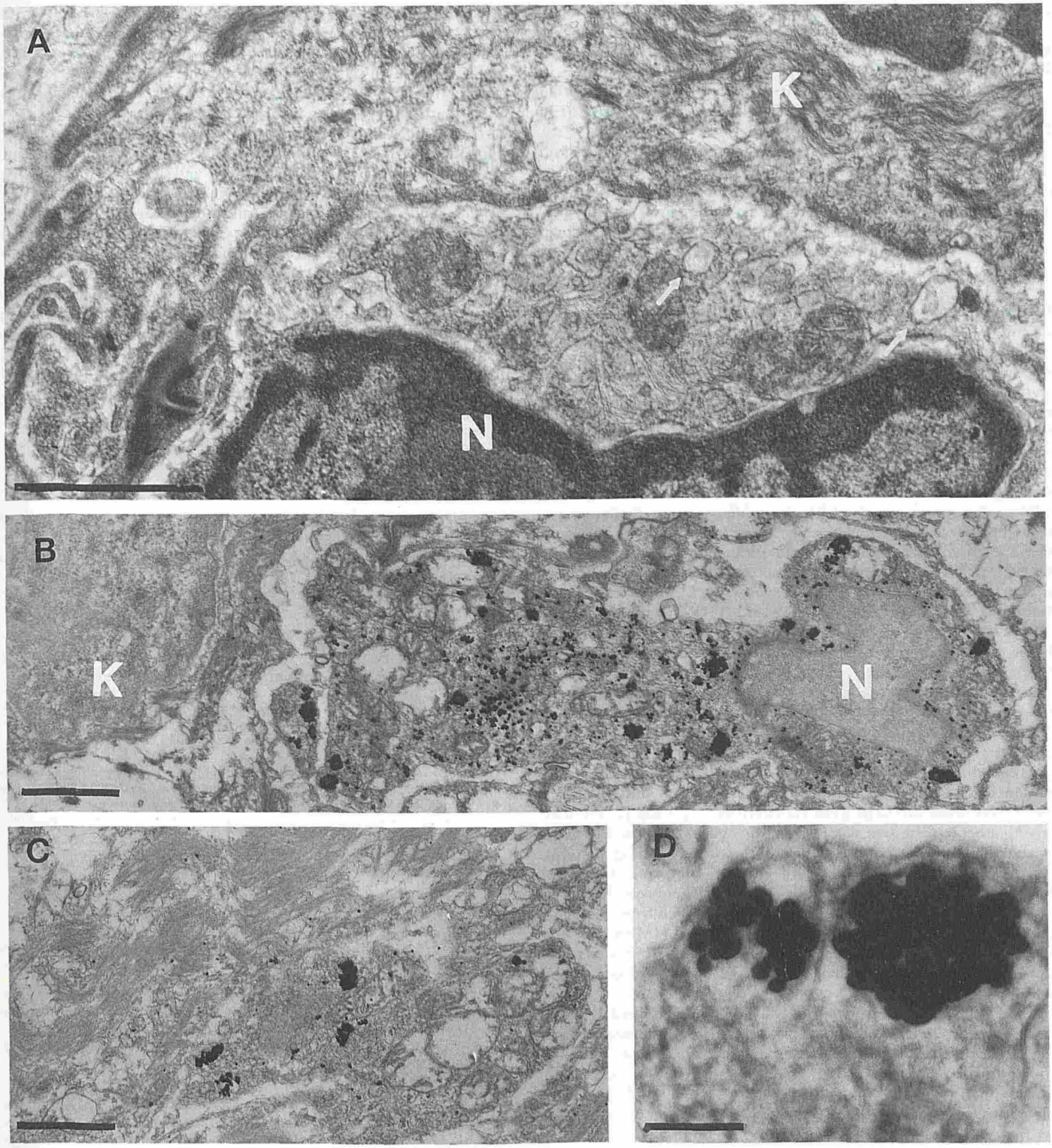

Figure 7. Electron microscopic analysis of plucked hairs sectioned and unlabeled (*A*), or labeled with an immunogold technique after incubation with NKI/beteb-antibody (*B,C, and D*). Ultrastructural analysis identified the NKI/beteb-positive cells as most compatible with ORS melanocytes. *A* shows unstained sections in which an ORS melanocyte (N, nucleus of ORS melanocyte) is adjacent to an ORS keratinocyte (K). The ORS melanocytes show various organelles in the cytoplasm including mitochondria and small vesicular structures consistent with premelanosomes (indicated by *small white arrows*). The adjacent keratinocytes show distinct keratin intermediate filaments. *B* shows a similar section from a specimen stained with NKI/beteb using an immunogold technique. The ORS melanocyte (again showing nucleus marked with N) contains aggregates of silver grains in large number. The neighboring keratinocyte has only single and sparse silver grains. A similar section (*C*) again contrasts the aggregates of silver grains in ORS melanocytes with the few grains in keratinocytes (note intermediate filaments). A higher power view of the aggregated silver grains (*D*) shows that some are present within membrane-bound structures presumed to be premelanosomes. In *A, B, and C*, the *bars*, 1 μm. In *D* the *bar*, 0.1 μm.

containing intermediate filaments (marker K). Small white arrows mark cytoplasmic organelles consistent with premelanosomes in this unstained specimen. In sections reacted with anti-NKI/beteb and labeled by an immunogold technique **(Fig 7*B*)**, there are multiple aggregates of gold particles in the cytoplasm of ORS melanocytes (nucleus marker with N) but not in surrounding

keratinocytes (marked by K). The cytoplasm of keratinocytes contains few single gold partricles in contrast to the aggregates of gold particles in ORS melanocytes incubated with NKI/beteb **(Fig 7*B,C*)**. A higher-power view of the aggregates of gold particles in ORS melanocytes shows that some of these aggregates are within membrane-bound structures consistent with premelanosomes **(Fig 7*D*)**.

Negative controls were also performed using the immunogold technique, either eliminating the primary antibody or substituting purified mouse IgG for the NKI/beteb 1. In both instances, the negative control experiments showed no aggregates of gold particles in any of the cells in the ORS, and showed only occasional single silver grains (data not shown).

DISCUSSION

We have clearly identified ORS melanocytes using monoclonal antibodies NKI/beteb and A4F11, which recognize premelanosome- and melanosome-related antigens of different molecular weights [14,15]. The staining pattern of the ORS melanocytes was granular and cytoplasmic. HMB45, which is an antibody to a melanosome-related cytoplasmic antigen [16], did not stain ORS melanocytes, although it did stain epidermal and hair bulb melanocytes. Thus, ORS melanocytes contain premelanosomes or premelanosome-related antigenic product(s), but not melanosome-related proteins. Lack of melanosomes but not premelanosomes in ORS melanocytes suggests a block in differentiation in these cells involving structural as well as enzymatic components of melanization. ORS melanocytes did not stain using antibodies to tyrosinase, TRP-1, or TRP-2. These are melanosomal glycoproteins that are known to be essential for melanin synthesis [12,13]. Melanocytes that stain with tyrosinase, TRP-1, and TRP-2 also react with DOPA and HMB45 and were seen in the epidermis and hair bulb. These results suggest that development of melanosomes from premelanosomes may be essential for the production of these melanosomal glycoproteins, or that melanosomal glycoprotein expression may be necessary for melanosome formation.

Recent studies using transfection techniques revealed that the antigen(s) recognized by NKI/beteb and HMB45 are the product(s) of the same gene, gp100-c1, which is homologous to the human silver locus gene, Pmel 17 [21,22]. Pmel 17 and gp100-c1 are derived from the same gene *via* alternative splicing [22]. Other investigators have shown that HMB45 recognizes the Pmel 17 gene product.¶ The difference in ORS melanocyte staining patterns between NKI/beteb (positive) and HMB45 (negative) might be due to differential glycosylation or alternative gene splicing. Our results support the hypothesis that, although derived from the same gene, the antigens recognized by NKI/beteb and HMB45 are not identical. Moreover, the antigen identified by NKI/beteb may be an antigen expressed in premelanosomes or lysosomes within inactive ORS melanocytes.

It has been well documented that DOPA or combined DOPA-premelanin positive melanocytes appear in the ORS during the hair peg stage of fetal development [23]. These DOPA-positive melanocytes then disappear in the bulbous peg stage; however, melanocytes that are DOPA-negative and combined DOPA/premelanin-positive exist until 6 months of fetal age. After 6 months of fetal age these cells are only seen in bulbar and/or infundibular portions. DOPA or DOPA/premelanin-positive cells were not seen in the ORS of human hair follicles in our experiments. It is likely that during the hair peg stage the cellular environment around melanocytes may provide suitable conditions for DOPA-positive ORS melanocytes. In hair follicles after 6 months of fetal age, these conditions are lost and then the ORS melanocytes become DOPA negative.

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It has been previously reported that senile white hairs contain ORS melanocytes [24]. We found a considerable number of NKI/beteb staining ORS melanocytes whereas none were found in the hair bulb of a senile white hair. Also, HMB45 did not stain any cells in the ORS or bulb of a senile white hair. It remains unknown if ORS melanocytes play a role in hair melanization.

DOPA-positive melanocytes appear in the ORS during the repigmenting phase of vitiligo induced by psoraten plus ultraviolet A [10,11] or other treatments [9], and are considered to be activated ORS melanocytes. The mechanisms of activation, proliferation, and migration of ORS melanocytes are still unclear. We have shown that ORS melanocytes contain early structural proteins (premelanosomes), but not later structural proteins (melanosomes) or enzymatic proteins (tyrosinase, TRP-1, TRP-2 proteins). We believe that activation of ORS melanocytes is the process whereby inactive ORS melanocytes are stimulated to synthesize all of the products necessary to allow for melanogenesis. Identification of the stimuli for this activation will be a key to developing specific new treatments for vitiligo. The results reported here will greatly facilitate studies of the activation of ORS melanocytes in the treatment of vitiligo.

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